I. In fact, Claims 18-21 depend directly (Claim 18) or indirectly (Claims 19-21) from Claim 1. Accordingly, since Claims 18-21 depend ultimately from the elected claims, should the claims of Group I be found allowable, then Claims 18-21 must be rejoined with Group I under the provisions of MPEP §821.04.

Applicants enclose a copy of reference AV listed in the IDS submitted on March 31, 2000. Consideration of this reference is respectfully requested.

The title of the application has been amended as suggested by the Examiner.

The rejection of the claims under 35 U.S.C. §101 is believed to be obviated by the amendment submitted above. The claims now recite "an isolated bacterium." Withdrawal of this ground of rejection is respectfully requested.

The rejection of the claims under 35 U.S.C. §112, second paragraph, is believed to be obviated by the amendment submitted above. Withdrawal of this ground of rejection is respectfully requested.

The rejection of the claims under 35 U.S.C. §112, first paragraph, is believed to be obviated by the amendment submitted above. Withdrawal of this ground of rejection is respectfully requested.

The rejection of Claims 1-4 and 8-10 under 35 U.S.C. §102(a) over Zakataeva et al. (IDS reference AY) is respectfully traversed. Zakataeva et al. is not available as prior art against the present application.

The Zakataeva et al. reference was published on June 11, 1999. The present application has a U.S. filing date of December 20, 1999 and claims priority to Russian patent application serial No. 98123511 (hereinafter referred to as RU '511). RU '511 was filed on December 23, 1998. An English translation of RU '511 is attached herewith.

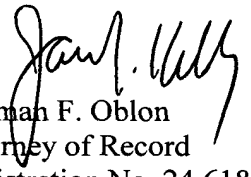
The pending claims of the present application are supported by the disclosure of RU '511 within the meaning of 35 U.S.C. §112. Since December 23, 1998 is prior to June 11, 1999, Zakataeva et al. is not available as prior art against the present application. Withdrawal of this ground of rejection is respectfully requested.

The rejection of Claims 1-4 and 8 under 35 U.S.C. §102(b) over Zakataeva et al. (IDS reference AZ) is believed to be obviated by the amendment submitted above. The rejection was based on the recitation (B) in the original claims (see the Official Action dated May 30, 2001 at paragraph 18 at pages 11-12). Recitation (B) is not present in the amended claims. Accordingly, withdrawal of this ground of rejection is respectfully requested.

Applicants submit that the application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.



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ATTORNEY DOCKET NO.: 0010-1070-0
SERIAL NO.: 09/466,935

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Serial No.: 09/466,935
Amendment Filed On: August 30, 2001

IN THE TITLE OF THE APPLICATION

Please replace the title with the following new title:

--NOVEL THREONINE RESISTANCE GENE--.

IN THE CLAIMS

Please cancel Claims 1-10.

Please add the following claims.

--11. (New)

12. (New)

13. (New)

14. (New)

15. (New)

16. (New)

17. (New)

18. (New)

19. (New)

20. (New)

21. (New)--

CHARACTERIZATION OF A PLEIOTROPIC MUTATION THAT CONFERS UPON *ESCHERICHIA COLI* CELLS RESISTANCE TO HIGH CONCENTRATIONS OF HOMOSERINE AND THREONINE.

N. P. Zakataeva, V. A. Aleoshin, and V. A. Livshits, State Institute of Genetics and Selection of Industrial Microorganisms, Moscow, Russia.

Homoserine, the precursor of both threonine and methionine, in high concentrations depresses the growth of different bacteria on minimal media. We obtained in *E. coli* K-12 a mutation, *rhtA23* (resistance to homoserine and threonine), which conferred resistance to high concentrations of homoserine (>5 mg/ml) or threonine (>40 mg/ml), and markedly improved the productivity of a threonine-producing strain. The mutation had multiple effects on cells. It changed their morphology, caused retardation of culture development, twice increased the levels of glutamate dehydrogenase and glutamate synthase. Further, the *rhtA23* mutation conferred increased resistance to lysine and histidine, as well as to some amino acid analogues. The *rhtA23* mutation was mapped at 18,3 min on the *E.coli* chromosome, and proved to be dominant.

Two types of inserts belonging to the different chromosome regions had been cloned from both *rhtA23* and wild type donors on the basis of their ability to confer homoserine and threonine resistance. Thus, at least two genes, *rhtA* and *rhtB*, exist in *E. coli* that in multicopy confer resistance to these amino acids. The *rhtB* gene is located at 86 min near *recQ gene*. *rhtA* gene was identified as ORF1 between *pexB* and *ompX* genes. In the *rhtA23* mutant A-for-G substitution was found at the position -1 with respect to the ATG start codon. The product of ORF1 is a hydrophobic protein of 295 amino acid residues with 8 predicted membrane-spanning regions. The *rhtA23* mutant accumulated in the culture broth three times more homoserine than the wild type strain. On the other hand, the intracellular homoserine concentration was 10-fold higher in the wild type strain than in the mutant. We think that RhtA protein is involved in homoserine and threonine efflux.

VERIFICATION OF TRANSLATION

Russian Patent Application No. 98123511
filed on December 23, 1998

I, Vitaliy Arkadyevich LIVSHITS , a citizen of Russia whose address is

kv. 84, korpus 1, Sumskoy proezd 5, Moscow, 113208, Russia

, am a translator of the document attached and I state that the following is a true translation to the best of my knowledge and belief.

Signature of translator:

Vitaliy Arkadyevich LIVSHITS

Signed at Moscow, Russia
This 30th day of August, 2001

RUSSIAN AGENCY FOR PATENTS AND TRADEMARKS
(ROSPATENT)

Federal Institute of Industrial Property

SERTIFICATE

Federal Institute of Industrial Property of the Russian Agency for Patents and Trademarks hereby confirms that the supplemented materials are a faithful reproduction of the initial description and figures of the patent application No. 98123511 filed December 23, 1998.

Designation of invention: A DNA fragment rhtC encoding RhtC protein which confers on bacterium Escherichia coli increased resistance to L-threonine, and a method for producing L-amino acids.

Declarant (s): State Research Institute for Genetics and Selection of Industrial Microorganisms (GNIIGenetika)

Actual inventors: Livshits, Vitaliy Arkadyevich
Zakataeva, Natalia Pavlovna
Aleshin, Vladimir Veniaminovich
Belareva, Alla Valentinovna
Tokhmakova, Irina Lyvovna

Authorized to certify the copy
of the patent application

G.F. Vostrikov
Head of the department

THE DNA FRAGMENT rhtC, ENCODING RhtC PROTEIN, WHICH CONFERS ON BACTERIUM ESCHERICHIA COLI RESISTANCE TO L-THREONINE, AND A METHOD FOR PRODUCING L-AMINO ACIDS

The present invention relates to biotechnology, and more specifically to a method for producing amino acid, especially for a method for producing L-homoserine, L-threonine, L-valine or L-leucine using a bacterium belonging to the genus Escherichia.

The present inventors obtained, with respect to E.coli K-12, a mutant having mutation, thrB (herein referred to as rhtA23) that is concerned in resistance to high concentrations of threonine or homoserine in a minimal medium (Astaurova, O.B. et al., Appl. Bioch. And Microbiol., 21, 611-616 (1985)). The mutation improved the production of L-threonine (SU Patent No. 974817), homoserine and glutamate (Astaurova, O.B. et al., Appl. Bioch. And Microbiol., 27, 556-561, 1991) by the respective E. coli producing strains.

Furthermore, the present inventors has revealed that the rhtA gene exists at 18 min on E.coli chromosome and that the rhtA gene is identical to ORF1 between pexB and ompX genes. The unit expressing a protein encoded by the ORF has been designated as rhtA (rht: resistance to

homoserine and threonine) gene. The rhtA gene includes a 5'-noncoding region including SD sequence, ORF1 and a terminator. Also, the present inventors have found that a wild type rhtA gene participates in resistance to threonine and homoserine if cloned in a multicopy state and that the rhtA23 mutation is an A-for-G substitution at position -1 with respect to the ATG start codon (ABSTRACTS of 17th International Congress of Biochemistry and Molecular Biology in conjugation with 1997 Annual Meeting of the American Society for Biochemistry and Molecular Biology, San Francisco, California August 24-29, 1997, abstract No. 457).

It is found that at least two different genes which impact threonine and homoserine resistance in a multicopy state exist in E.coli during cloning of the rhtA gene. One of the genes is the rhtA gene, and the other gene was found to be rhtB gene which confers homoserine resistance (Russian Patent Application No. 98118425).

An object of the present invention is to provide a a method for producing an amino acid, especially, L-homoserine, L-threonine and branched chain amino acids with a higher yield.

The goal was achieved by obtaining of a DNA fragment rhtC encoding the RhtC protein which confers upon

bacterium E. coli increased resistnace to L-threonine, and by construction on the basis of this fragment of a new strain which permits to produce an amino acid with a higher yield.

Thus, the present invention provides:

(1) A bacterium belonging to the genus Escherihia, wherein L-threonine resistance of the bacterium is enhanced by enhancing an activity of protein as defined in the following (A) or (B) in a cell of the bacterium:

(A) a protein which comprises an amino acid sequence shown in Fig.2; or

(B) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence shown in Fig.2, and which has an activity of making a bacterium having the protein L-threonine-resistant;

(2) The bacterium according to (1), wherein L-homoserine resistance of the bacterium is further enhanced by enhancing an activity of protein as defined in the following (C) or (D) in a cell of the bacterium:

(C) a protein which comprises an amino acid sequence shown in Fig.4; or

(D) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence

shown in Fig.4, and which has an activity of making a bacterium having the protein L-homoserine-resistant;

(3) The bacterium according to (1) or (2), wherein the activity of protein as defined in (A) or (B) is enhanced by transformation of the bacterium with DNA coding for the protein as defined in (A) or (B);

(4) The bacterium according to (2), wherein the activity of protein as defined in (C) or (D) is enhanced by transformation of the bacterium with DNA coding for the protein as defined in (C) or (D);

(5) A method for producing an amino acid, comprising the steps of:

cultivating the bacterium as defined in any one of (1) to (4), which has an ability to produce the amino acid, in a culture medium, to produce and accumulate the amino acid in the medium, and recovering the amino acid from the medium;

(6) The method according to (5), wherein the amino acid is selected from the group consisting of L-homoserine, L-threonine and branced chain amino acids; and

(7) The method according to (6), the branced chain amino acid is L-valine or L-leucine.

The DNA fragment coding for the protein as defined in the above (A) or (B) may be referred to as «rhtC

gene», a protein coded by the rhtC gene may be referred to as «RhtC protein», the DNA coding for the protein as defined in the above (C) or (D) may be referred to as «rhtB gene», a protein coded by the rhtB gene may be referred to as «RhtB protein». An activity of the RhtC protein which participate in resistance to L-threonine of a bacterium (i.e. an activity of marking a bacterium having the RhtC protein L-threonine-resistant) may be referred to as «Rt activity», and an activity of the RhtB protein which participates in resistance to L-homoserine of a bacterium (i.e. an activity of marking a bacterium having the RhtB protein L-homoserine-resistant) may be referred to as «Rh activity». A structural gene encoding the RhtC protein or RhtB protein in the rhtC gene or rhtB gene may be referred to as «rhtC structural gene» or «rhtB structural gene». The term «enhancing the Rt activity or the Rh activity» means imparting resistance to threonine or homoserine to a bacterium or enhance the resistance by means of increasing the number of molecules of the RhtC protein or RhtB protein increasing a specific activity of these proteins, or desensitizing negative regulation against the expression or the activity of these proteins or the like. The terms «DNA coding for a protein» mean a DNA of which one of strands codes for the protein when the DNA

is double-stranded. The L-threonine resistance means a property that a bacterium grows on a minimal medium containing L-threonine at a concentration at which a wild-type strain thereof not grow, usually at >30 mg\ml. The L-homoserine resistance means a property that a bacterium grows on a minimal medium containing L-homoserine at a concentration at which a wild-type strain thereof not grow, usually at >5 mg\ml. The ability to produce an amino acid means a property that a bacterium produce and accumulates the amino acid in a medium in a larger amount than a wild type strain thereof.

According to the present invention, resistance to threonine, or threonine and homoserine of a high concentration can be imparted to a bacterium belonging to the genus Escherichia. A bacterium belonging to the genus Escherichia, which has increasing resistance to threonine, or threonine and homoserine and an ability to accumulate an amino acid, especially, L-homoserine, L-threonine, or branced chain amino acids such as L-valin and L-leucine in a medium with a high yield.

The present invention will be explained in detail below.

The first DNA fragment used for the present invention (rhtC gene) coding for a protein having the Rt

activity and having an amino acid sequence shown in Fig.2. Specifically, the DNA may be exemplified by a DNA comprising a nucleotide sequence of the nucleotide numbers 187 to 804 of a nucleotide sequence shown in in Sequence No.1.

The second DNA fragment used for the present invention (rhtB gene) coding for a protein having the Rh activity an having an amino acid sequence shown in Fig.4. Specifically, the DNA may be exemplified by a DNA comprising a nucleotide sequence of the nucleotide numbers 557 to 1171 of a nucleotide sequence shown in Fig.3 (Sequence No.3).

The rhtB gene having the nucleotide sequence shown in Fig.3 corresponds to a part of sequence complement to the sequence of GenBank accession number M87049, and includes f138 (nucleotide numbers 61959-61543 of M87049) which is a known but function-unknown ORF (open reading frame) present at 86 min on E.coli chromosome, and 5'- and 3'- flanking regions thereof. The f138, which had only 160 nucleotides in the 5'-flanking region, could not impart the resistance to homoserine. No termination codon is present between the 62160 and 61959 nucleotides of M87049 (upstream the ORF f138). Moreover, one of the ATG codons of this sequence is preceded by a ribosome-binding site (62171-62166 in M87049). Hence, the coding

region is 201 bp longer. The larger ORF (nucleotide numbers 62160 to 61546 of M87049) is designated as rhtB gene.

The rhtB gene may be obtained, for example, by infecting Mucts lysogenic strain of E.coli using a lysate of a lysogenic strain of E.coli such as K12 or W3110 according to the method in which mini-Mu d5005 phagemid is used (Groisman, E.A., et al., J. Bacteriol., 168, 357-364 (1986)), and isolating phagemid DNAs from colonies growing on a minimal medium containing kanamycin (40 μg\ml) and L-homoserine (10 mg|ml). As illustrated in the Example described below, the rhtB gene was mapped at 86 min on the chromosome of E.coli. Therefore, the DNA fragment including the rhtB gene may be obtained from the chromosome of E.coli by colony hybridization or PCR (polymerase chain reaction, refer to White, T.J. et al, Trends Genet. 5, 185 (1989))using oligonucleotide(s) which has a sequence corresponding to the region near the portion of 86 min on the chromosome E.coli.

Alternatively, the oligonucleotide may be designed according to the nucleotide sequence shown in Fig.3. By using oligonucleotides having nucleotide sequences corresponding to an upstream region from the nucleotide number 557 and a downstream region from the nucleotide

number 1171 in Fig.3 as the primers for PCR, the entire coding region can be amplified.

Synthesis of the oligonucleotides can be performed by an ordinary method such as a phosphoamidite method (see Tetrahedron Letters, 22, 1859 (1981)) by using a commercially available DNA synthesizer (for example, DNA Synthesizer Model 380B produced by Applied Biosystems). Further, the PCR can be performed by using a commercially available PCR apparatus (for example, DNA Thermal Cycler Model PJ2000 produced by Takara Shuzo Co., Ltd.) using Taq DNA polymerase (supplied by Takara Shuzo Co., Ltd.) in accordance with a method designate by the supplier.

The rhtC gene was obtained in the DNA fragment including rhtB gene by chance when rhtB was cloned as described later in the embodiments. The rhtC gene corresponds to a corrected, as described below, sequence of O128,(nucleotide numbers 60860-61480 of GeneBank accession number M87049) which is a known but function-unknown ORF. The rhtC gene may be obtained by PCR or hybridization using oligonucleotides designed according to the nucleotide sequence No.1 (Claim 1). By using olidonucleotides having nucleotide sequence corresponding to a upstream region from nucleotide number 187 and a downstream region from the nucleotide

number 804 in sequence No.1 as the primers for PCR, the entire coding region can be amplified.

In the present invention, the DNA coding for the RhtB protein of the present invention may code for RhtB protein including deletion, substitution, insertion, or addition of one or several amino acids at one or a plurality of positions, provided that the Rh activity of RhtB protein encoded thereby is not deteriorated. Similarly, the DNA coding for the RhtC protein of the present invention may code for RhtC protein including deletion, substitution, insertion, or addition of one or several amino acids at one or a plurality of positions, provided that the Rt activity of RhtC protein encoded thereby is not deteriorated.

The DNA, which codes for the substantially same protein as the RhtB protein or RhtC protein as described above, may be obtained, for example, by modifying the nucleotide sequence, for example, by means of the site-directed mutagenesis method so that one or more amino acid residues at a specified site involve deletion, substitution, insertion, or addition. DNA modified as described above may be obtained by the conventionally known mutation treatment. The mutation treatment includes a method for treating a DNA coding for the RhtB protein or RhtC protein in vitro, for example, with

hydroxylamine, and a method for treating a microorganism, for example, a bacterium, belonging to the genus Escherichia harboring a DNA coding for the RhtB protein with ultraviolet irradiation or a mutating agent such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and nitrous acid usually used for the mutation treatment.

The DNA, which codes for substantially the same protein as the RhtB protein or RhtC protein, can by obtained by expressing a DNA subjected to in vitro mutation treatment as described above in multicopy in an appropriate cell, investigating the resistance to homoserine or threonine, and selecting the DNA which increase the resistance.

It is generally known that an amino acid sequence of a protein and a nucleotide sequence coding for it may be slightly different between species, strains, mutants or variants.

Therefore the DNA, which codes for substantially the same protein as the RhtC protein, can be obtained by isolating a DNA which hybridizes with DNA having, for example, a nucleotide sequence of the nucleotide numbers 187 to 804 of the nucleotide sequence shown in sequence No.1 (see Claim 1) under stringent conditions, and which codes for a protein having the Rt activity from a bacterium belonging to the genus Escherihia which is

subjected to mutation treatment, or a spontaneous mutant or a variant of a bacterium belonging to the genus Escherihia.

Also, the DNA, which codes for substantially the same protein as the RhtB protein, can be obtained by isolating a DNA which hybridizes with DNA having, for example, a nucleotide sequence of the nucleotide numbers 557 to 1171 of the nucleotide sequence shown in Fig.3 under stringent conditions, and which codes for a protein having the Rh activity, from a bacterium belonging to the genus Escherichia which is subjected to mutation treatment, or a spontaneous mutant or a variant of a bacterium belonging to the genus Escherichia.

The term "stringent conditions" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent conditions include a condition under which DNAs having high homology, for example, DNAs having homology of not less than 70% with each other are hybridized, and DNAs having homology lower than the above with each other are not hybridized.

The bacterium belonging the genus Escherichia of the present invention is a bacterium belonging to the genus

Escherichia of which the Rt activity is enhanced. Preferred embodiment of the bacterium of the present invention is a bacterium which is further enhanced the Rt activity. A bacterium belonging to the genus Escherichia is exemplified by Escherichia coli. The Rt activity can be enhanced by, for example, amplification of the copy number of the rhtC structural gene in a cell, or transformation of a bacterium belonging to the genus Escherihia with a recombinant DNA in which a DNA fragment including the rhtC structural gene encoding the RhtC protein is ligated with a promoter sequence which functions efficiently in a bacterium belonging to the genus Escherihia. The Rt activity can be also enhanced by substitution of the promoter sequence of the rhtC gene on a chromosome with a promoter sequence which functions efficiently in a bacterium belonging to the genus Escherichia.

Besides, the Rh actibity can be enchanced by, for example, amplification of the copy number of the rhtB structural gene in a cell, or transformation of a bacterium belonging to the genus Escherichia with recombinant DNA in which a DNA fragment including the rhtB structural gene encoding RhtB protein is ligated with a promoter sequence which functions efficiently in a bacterium belonging to the genus Escherichia. The Rh

activity can be also enhanced by substitution of the promoter sequence of the rhtB gene on a chromosome with a promoter sequence which functions efficiently in a bacterium belonging to the genus Escherichia.

The amplification of the copy number of the rhtC structural gene or rhB structural gene in a cell can be performed by introduction of a multicopy vector which carries the rhtC structural gene or rhtB structural gene into a cell of a bacterium belonging to the genus Escherihia. Specifically, the copy number can be increased by introduction of a plasmid, a phage or a transposon (Berg, D.E. and Berg, C.M., Bio\Tecnol., 1, 417 (1983)) which carries the rhtC structural gene or rhtB structural gene into a cell of a bacterium belonging to the genus Escherichia.

The multicopy vector is exemplified by plasmid vectors such as pBR322, pMW118, pUC19 or the like, and phage vectors such as λ1059, λBF101, M13mp9 or the like. The transposon is exemplified by Mu, Tn10, Tn5 or the like.

The introduction of a DNA into a bacterium belonging to the genus Escherichia can be performed, for example, by a method of D.M.Morrison (Methods in Enzymology, 68, 326 (1979)) or a method in which recipient bacterial cell are treated with calcium chloride to increase

permeability of DNA (Mandel, M. And Higa, A., J. Mol. Biol., 53, 159, (1970)) and the like.

If the Rt activity, or the Rt activity and the Rh activity is enhanced in an amino acid-producing bacterium belonging to the genus Escherichia as described above, a produced amount of the amino acid can be increased. As the bacterium belonging to the genus Escherichia which is to be the Rt activity, or the Rt activity and the Rh activity is enhanced, strains which have abilities to produce desired amino acids are used. Besides, an ability to produce an amino acid may be imparted to a bacterium in which the Rt activity, of the Rt activity and Rh activity is enhanced.

On the basis of the rhtC DNA fragment amplification the new strains E. coli MG442/pRhtC producing homoserine; E. coli MG442/pVIC40,pRhtC producing threonine; E. coli NZ10/pRhtBC and E. coli NZ10/pRhtB, pRhtC producing homoserine, valine and leucine were obtained which accumulate the amino acids in a higher amount than those containing no amplified rhtC DNA fragment.

The new strains have been deposited (according to international deposition based on Budapest Treaty) in the All-Russian Collection for Industrial Microorganisms (VKPM). The strain E. coli MG442/pRhtC has been

deposited as an accession number of VKPM B-7700; the strain E. coli MG442/pVIC40,pRhtC has been deposited as an accession number of VKPM B-7680; the strain E. coli NZ10/pRhtB, pRhtC has been deposited as an accession number of VKPM B-7681, and the strain E. coli NZ10/pRhtBC has been deposited as an accession number of VKPM B-7682.

The strain E. coli MG442/pRhtC (VKPM B-7700) exhibits the following cultural-morphological and biochemical features.

Cytomorphology. Gram-negative weakly-motile rods having rounded ends. Longitudinal size, 1.5 to 2 μm.

Cultural features.

Beef-extract agar. After 24 hours of growth at 37° C. produces round whitish semitransparent colonies 1.0 to 3 mm in diameter, featuring a smooth surface, regular or slightly wavy edges, the centre is slightly raised, homogeneous structure, pastelike consistency, readily emulsifiable.

Luria's agar. After a 24-hour growth at 37° C. develops whitish semitranslucent colonies 1.5 to 2.5 mm in

diameter having a smooth surface, homogeneous structure, pastelike consistency, readily emulsifiable.

Minimal agar-doped medium of Adams. After 40 to 48 hours of growth at 37°C forms colonies 0.5 to 1.5 mm in diameter, which are coloured greyish-white, semitransparent, slightly convex, with a lustrous surface.

Growth in a beaf-extract broth. After a 24-hour growth at 37° C exhibits strong uniform cloudiness, has a characteristic odour.

Physiological and biochemical features.

Grows upon thrust inoculation in a beef-extract agar. Exhibits good growth throughout the inoculated area. The microorganism proves to be a facultative anaerobe.

It does not liquefy gelatin.

Features a good growth on milk, accompanied by milk coagulation.

Does not produce indole.

Temperature conditions. Grows on beaf-extract broth at 20 - 42° C, an optimum temperature lying within 33-37 ° C.

pH value of culture medium. Grows on liquid media having the pH value from 6 to 8, an optimum value being 7.2.

Carbon sources. Exhibits good growth on glucose,

fructose, lactose, mannose, galactose, xylose, glycerol, mannitol to produce an acid and gas.

Nitrogen sources. Assimilates nitrogen in the form of ammonium, nitric acid salts, as well as from some organic compounds.

Resistant to ampicillin.

Content of plasmids. The cells contain multicopy hybride plasmid pRhtC ensuring resistance to ampicillin (100 mg/l) and carrying the rhtC gene responsible for the increased resistance to threonine (50 mg/ml).

The strain E. coli MG442/pVIC40, pRhtC (VKPM B-7680) has the same cultural-morphological and biochemical features as the strain VKPM B-7700 except for in addition to pRhtC it contains a multicopy hybride plasmid pVIC40 ensuring resistance to streptomycin (100 mg/l) and carrying the genes of the threonine operon.

The strain E. coli strain E. coli NZ10/pRhtB, pRhtC (VKPM B-7681) has the same cultural-morphological and biochemical features as the strain VKPM B-7700 except for L-threonine (0.1 - 5 mg/ml) is used as a growth factor instead of L-isoleucine. Besides, it contains a multicopy hybride plasmid pRhtB ensuring resistance to kanamycin (50 mg/l) and carrying the rhtB gene which

confers resistance to homoserine (10 mg/ml)

The strain E. coli strain E. coli NZ10/pRhtBC, (VKPM B-7682) has the same cultural-morphological and biochemical features as the strain VKPM B-7681 except for it contains a multicopy hybride plasmid pRhtBC ensuring resistance to ampicillin and carrying both the rhtB and rhtC genes.

Method for producing an amino acid

An amino acid can be efficiently produced by cultivating the bacterium in which the Rt activity, or the Rt activity and Rh activity is enhanced by amplifying a copy number of the rhtC gene, or rhtC gene and rhtB gene as describe above, and which has an ability to produce the amino acid, in a culture medium, producing and accumulating the amino acid in the medium, and recovering the amino acid from the medium. The amino acid is exemplified preferably by L-homoserine, L-threonine and branched chain amino acids. The branched chain amino acids may be exemplified by L-valine, L-leucine and L-isoleucine, and preferably exemplified by L-valine, L-leucine.

In the method of present invention, the cultivation of the bacterium belonging to the genus Escherichia, the

collection and purification of amino acids from the liquid medium may be performed in a manner similar to those of the conventional method for producing an amino acid by fermentation using a bacterium. A medium used in cultivation may be either a synthetic medium or a natural medium, so long as the medium includes a carbon and a nitrogen source and minerals and, if necessary, nutrients which the bacterium used requires for growth in appropriate amount. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. Depending on assimilatory ability of the used bacterium. Alcohol including ethanol and glycerol may be used. As the nitrogen source, ammonia, various ammonium salts as ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean hydrolyzate and digested fermentative microbe are used. As minerals, monopotassium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium carbonate are used.

The cultivation is preferably culture under an aerobic condition such as a shaking, and an aeration and stirring culture. The temperature of culture is usually 20 to 40°C, preferably 30 to 38°C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and

7.2. the pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, a 1 to 3-day cultivation leads to the accumulation of the target amino acid in the medium.

Recovering the amino acid can be performed by removing solids such as cells from the medium by centrifugation or membrane filtration after cultivation, and then collecting and purifying the target amino acid by ion exchange, concentration and crystalline fraction methods and the like.

Brief Explanation of Drawings

Fig 1. shows cloning and identification of rhtB and rhtC genes,

Fig 2. shows the amino acid sequence of RhtC protein,

Fig 3 shows the nucleotide sequence of the DNA fragment containing the rhtB gene.

Fig 4. shows the amino acid sequence of RhtB protein,

Fig 5. shows structure of the plasmid pRhtB harboring rhtB gene,

Fig 6. shows structure of the plasmid pRhtC harboring rhtC gene, and

Fig 7. shows structure of the plasmid pRhtBC harboring rhtB gene and rhtC gene.

The present invention will be more concretely explained below with reference to Examples. In the Examples, an amino acid is of L-configuration unless otherwise noted.

Example 1: Obtaining of the rhtB and rhtC DNA fragments

Step 1. Cloning of genes involving resistance homoserine and threonine into mini-Mu phagemid

The genes involving resistance homoserine and threonine were cloned *in vivo* using mini-Mu d5005 phagemid (Groisman, E.A., et al., J. Bacteriol., 168, 357-364 (1986)). MuCts62 lysogen of the strain MG442 (Guayatiner et al., Genetika (in Russian), 14, 947-956 (1978)) was used as a donor. Freshly prepared lysated were used to infect a Mucts lysogenic derivative of a strain VKPM B-513 (Hfr K10 metB). The cells were plated on M9 glucose minimal medium with methionine (50 μg/ml), kanamycin (40 μg/ml) and homoserine (10 μg/ml) and cultured at 30°C. Colonies which appeared after 48 hr were picked and isolated. Plasmid DNA was isolated and used to transform the strain VKPM B-513 by standard techniques. Transformants were selected on L-broth agar plates with kanamycun as above. Plasmid DNA was isolated from those which were resistance to homoserine, and

analyzed by restriction mapping of the structure of the inserted fragments. It appeared that two types of inserts belonging to different chromosome regions had been cloned from the donor. Thus, at least two different genes that in multicopy impart resistance to homoserine exist in E.coli. One of the two types of inserts is the rhtA gene which has already reported (ABSTRACT of 17th International Congress of Biochemistry and Molecular Biology in conjugation with 1997 Annual Meeting af the American Society for Biochemistry and Molecular Biology, San Francisco, California August 24-29, 1997). Among the other of the two types of inserts, a MluI-MluI fragment of 0.8 kb imparts only the resistance to homoserine (Fig. 1).

Step 2: Identification of rhtB gene and rhtC gene

The insert MluI-MluI fragment was sequenced by the dideoxy chain termination method of Sanger. Both DNA strands were sequenced in their entirety and all junctions were overlapped. The sequencing showed that the insert fragment included f138 (nucleotide numbers 61543 to 61959 of GenBank accession number M87049) which was a known but function-unknown ORF(open reading frame) present at 86 min of E.coli chtomosome and about 201 bp of an upstream region thereof (downstream region in the

sequence of M87049). The f138 which had only 160 nucleotides in the 5'-flanking region could not impart the resistance to homoserine. No termination codon is present upstream the ORF f138 between 62160 and 61950 nucleotides of M87049. Furthermore, one ATG following a sequence predicted as a ribosome binding site is present in the sequence. The larger ORF (nucleotide numbers 62160 to 61546) is designated as rhtB gene. The RhtB protein deduced from the gene has a region which is highly hydrophobic and contais possible transmembrane segments.

As described below, the plasmid containing this gene conferred upon cells only the resistance to high concentrations of homoserine. Since the initial SacII-SacII DNA fragment contained the second unidentified ORF, 0128, the gene was subcloned and tested for its ability to confer resistance to homoserine and threonine. It proved that the plasmid containing 0128 (ClaI-Eco47III fragment) conferred resistance to 50 mg/ml threonine (Fig. 1). The subcloned fragment was sequenced and found to contain additional nucleotide (G) in the position between 61213 and 61214 nucleotides of M87049. The nucleotide addition to the sequence eliminated a frame shift and enlarged the ORF into 5'-flanking region up to 60860 nucleotide. This new gene was designated as rhtC.

Both genes, rhtB and rhtC, were found to be homologous to transporter involved in lysine export of Corynebacterium glutamicum.

Example 2: The effect of rhtB and rhtC genes amplification on homoserine production.

<1> Construction and homoserine production by the L-homoserine -producing strain E. coli NZ10/pAL4, pRhtB

The rhtB gene was inserted to a plasmid pUK21 (Vieira, J. And Messing, J., Gene, 100, 189-194 (1991)), to obtain pRhtB (Fig. 5).

Strain NZ10 of E.coli was transformed by a plasmid pAL4 which was a pBR322 vector into which the thrA gene coding for aspartokinase-homoseine dehydrogenase I was inserted, to obtain the strains NZ10/pAL4. The strain NZ10 is a leuB$^+$-reverted mutant thrB$^-$ obtained from the E.coli strain C600 (thrB, leuB) (Appleyard R.K., Genetics, 39, 440-452,1954).

The strain NZ10/pAL4 was transformed with pUK21 or pRhtB to obtain strains NZ10/pAL4,pUK21 and NZ10/pAL4, pRhtB.

The thus obtained transformants were each cultivated at 37°C for 18 hours in LB broth (Miller, J.H. Experiments in Molecular Genetics. 1972. Cold Spring Harbor Laboratory) with 50 mg/l kanamycin and 100 mg/l

ampicilin, and 0.3 ml of the obtained culture was inoculate into 3 ml of a fermentation medium having the following composition and containing 50 mg/l kanamycin and 100 mg/l ampicilin, in a 20 x200 mm test tube, and cultivated at 37°C for 48 hours with a rotary shaker. After the cultivation, an accumulated amount of homoserine in the medium and an an absorbance at 560 nm of the medium were determined by known methods.

[Fermentation medium composition (g/L)]

Glucose	80
$(NH_4)_2SO_4$	22
K_2HPO_4	2
NaCl	0.8
$MgSO_4*7H_2O$	0.8
$FeSO_4*7H_2O$	0.02
$MnSO_4*5H_2O$	0.02
Thiamine hydrochloride	0.2
Yeast Extract	1.0
$CaCO_3$	30

($CaCO_3$ was separately sterilized)

The results are shown in Table 1. As shown in Table 1, the strain NZ10/pAL4,pRhtB accumulated homoserine in

a larger amount than the strain NZ10/pAL4,pUK21 in which the rhtB gene was not enhanced.

Table 1.

Strain	OD_{560}	Accumulated amount of homoserine, g/L)
NZ10/pAL4,pUK21	14.3	3.3
NZ10/pAL4,pRhtB	15.6	6.4

<2> Construction and homoserine production by the homoserine-producing strain E. coli MG442/pRhtC

The rhtC gene was inserted to pUC21 vector (Vieira, J. And Messing, J., Gene, 100, 189-194 (1991)), to obtain pRhtC (Fig. 6).

The known E.coli strain MG442 (Gusyatiner, et al., 1978, Genetika (in Russian), 14:947-956) was transformed by introducing pUC21 or pRhtC to obtain the strains MG422/pUC21 and MG422/pRhtC.

The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 100 mg/ml ampicilin, and 0.3 ml of the obtained culture was inoculate into 3 ml of a fermentation medium describe above and containing 100 mg/ml ampicilin, in a 20 x 200 mm test tube, and cultivated at 37°C for 48 hours with a rotary shaker. After the cultivation, an accumulated

amount of homoserine in the medium and an an absorbance at 560 nm of the medium were determined by known methods. The results are shown in Table 2.

Table 2.

Strain	OD_{560}	Accumulated amount of homoserine (g/L)
MG422/pUC21	9.7	<0.1
MG422/pRhtC	15.2	9.5

Example 3: The effect of rhtB and rhtC genes amplification on threonine production.

<1> Construction and threonine production by the threonine -producing strain E. coli VG442/pVIC40, pRhtB (VKPM B-7660)

The strain MG442 was transformed by introducing a known plasmid pVIC40 (U.S. Patent No. 5,175,107 (1992)) by an ordinary transformation method. Transformants were selected on LB agar plates containing 0.1 mg/ml streptomycin. Thus a novel strain MG422/pVIC40 was obtained.

The strain MG422/pVIC40 was transformed with pUK21 or pRhtB to obtain strain MG422/pVIC40,pUK21 and MG422/pVIC40,pRhtB.

The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/l

kanamycin and 100 mg/l streptomycin, and 0.3 ml of the obtained culture was inoculate into 3 ml of a fermentation medium describe in Example 2 and containing 50 mg/l kanamycin and 100 mg/l streptomycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 68 hours with a rotary shaker. After the cultivation, an accumulated amount of threonine in the medium and an an absorbance at 560 nm of the medium were determined by known methods.

The results are shown in Table 3. As shown in Table 3, the strain MG422/pVIC40,pRhtB accumulated threonine in a larger amount than the strain MG422/pVIC40,pUK21 in which the rhtB gene was not enhanced.

Table 3.

Strain	OD_{560}	Accumulated amount of threonine (g/L)
MG422/pVIC40,pUK21	16.3	12.9
MG422/pVIC40,pRhtB	15.2	16.3

<2> Construction and threonine production by the threonine -producing strain E. coli VG442/pVIC40, pRhtC (VKPM B-7680)

The strain MG422/pVIC40 was transformed with pRhtC and pUC21. Thus the transformants MG422/pVIC40,pRhtC and

MG442/pVIC40, pUC21 were obtained. In the sane manner as describe above, MG422/pVIC40,pUC21 and MG422/pVIC40,pRhtC were each cultivated at 37°C for 18 hours in a nutrient broth with 100 mg/l ampicilin and 100 mg/l streptomycin and 0.3 ml of the obtained culture was inoculate into 3 ml of a fermentation medium describe above and containing 100 mg/l ampicilin and 100 mg/l streptomycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 46 hours with a rotary shaker. After the cultivation, an accumulated amount of threonine in the medium and an an absorbance at 560 nm of the medium were determined by known methods.

The results are shown in Table 4. As shown in Table 4, the strain MG422/pVIC40,pRhtC accumulated threonine in a larger amount than the strain MG422/pVIC40,pUC21 in which the rhtC gene was not enhanced.

Table 4

Strain	OD_{560}	Accumulated amount of threonine (g/L)
MG422/pVIC40, pUC21	17.4	4.9
MG422/pVIC40,pRhtC	15.1	10.2

As shown in Table 4, the strain MG442/pVIC40,pRhtC accumulated threonine in a larger amount than the strain

MG422/pVIC40,pUC21 in which the rhtC gene was not enhanced.

Example 4: Concerted effect of rhtB gene and rhtC gene on amino acid production

The SacII-SacII DNA fragmentcontaining both rhtB and rhtC genes was inserted to the pUC21 Thus the plasmid pRhtBC was obtained which harbors the rhtB gene and rhtC gene.

Then, the strain NZ10 was transformed with pUC21, pRhtB, pRhtC or pRhtBC, and the transformants NZ10/pUC21 (VKPM B-7685), NZ10/pRhtB (VKPM B-7683), NZ10/pRhtC (VKPM B-7684), NZ10/pRhtB, pRhtC (VKPM B-7681) and NZ10/pRhtBC (VKPM B-7682) were thus obtained.

The transformants obtained above were cultivated as the same manner as describe above and accumulated amounts of various amino acids in the medium and an absorbance at 540 nm of the medium were determined by known methods.

The result were shown in Table 5. It follows from the Table 5 that concerted effect of the pRhtB and pRhtC on producrion of homoserine, valine and leucine. These results indicate that the rhtB and rhtC gene products may interact in cells.

Table 5.

Strain	OD_{550}	Homoserine (g/L)	Valine (g/L)	Leucine (g/L)
NZ10/pUC21	18.7	0.6	0.22	0.16
NZ10/pRhtB	19.6	2.3	0.21	0.14
NZ10/pRhtC	20.1	0.7	0.2	0.15
NZ10/pRhtBC	21.8	4.2	0.34	0.44
NZ10/pRhtB,pRhtC	19.2	4.4	0.35	0.45

Example 5: Effect of rhtB gene and rhtC gene on resistance to amino acids

As describe above, the plasmids harboring the rhtB and rhtC have positive effect on some amino acid accumulation in culture broth by different strains. It proved that the pattern of accumulated amino acid was dependent on the strain genotype. The homology of the rhtB and rhtC genes products with the lysine transporter LysE of Corynebacterium glutamicum (Vrljic, M., Sahm, H. and Eggeling, L. (1996) Mol. Microbiol. 22, 815-826.) indicates the analogues function for these proteins.

Therefore, the effect of the pRhtB and pRhtC plasmids on susceptibility of the strain N99 which is a streptomycin-resistant (Str^R) mutant of the known strain W3350 (VKPM B-1557) to some amino acids and amino acid analogues was tested. Overnight broth cultures (10^9

cfu/ml) of the strains N99/pUC21, N99pUK21, N99/pRhtB and N99/pRhtC were diluted 1:100 in M9 minimal medium and grown for 5 h in the same medium. Then the log phase cultures thus obtained were diluted and about 10^4 viable cells were applied to well-dried test plates with M9 agar (2%) containing doubling increments of amino acids or analogues. Thus the minimum inhibitory concentration (MIC) of these compounds were examined.

The result are shown in Table 6. It follows from the Table 6 that multiple copies of rhtB besides homoserine confered increased resistance to α-amino-β-hydroxyvaleric-acid (AHVA) and S-(2-aminoethyl)-L-cysteine (AEC), and 4-aza-DL-leucine; and multiple copies of rhtC gene besides threonine increased resistance to valine, histidine, and AHVA. This results indicates that every of the presumed transporters, RhtB and RhtC, have specificity to several substrates (amino acids), or may shown non-specific effects as a result of amplification.

Table 6.

Substrate	MIC (μg/ml)		
	N99/pUC21*	N99/pRhtB	N99/pRhtC
L-homoserine	1000	20000	1000

L-threonine	30000	40000	80000
L-valine	0.5	0.5	2.0
L-histidine	5000	5000	40000
AHVA	100	2000	15000
AEC	5	20	5
4-aza-DL-leucine	50	100	50
O-methyl-L-threonine	20	20	20

*: The same data were obtain with N99/pUK21-

What is claimed is:

1. A DNA fragment, rhtC, encoding the RhtC protein which confers bacterium Escherihia coli increased resistance to L-threonine, containing the regulatory elements of rhtC gene and the structural part of rhtC gene and having the following nucleotide sequence: {SEQ ID NO: 3 in Sequence Listing]:

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atgccgatca ccgccagcga aatgctcagc gttaacggcg ttgggatgcg caagctggaa 60
cgctttggca aaccgtttat ggcgctgatt cgtgcgcatg ttgatggcga tgacgaagag 120
tagtcagcag cataaaaaag tgccagtatg aagactccgt aaacgtttcc cccgcgagtc 180
aaatgt atg ttg atg tta ttt ctc acc gtc gcc atg gtg cac att gtg    228
       Met Leu Met Leu Phe Leu Thr Val Ala Met Val His Ile Val
         1               5                  10
gcg ctt atg agc ccc ggt ccc gat ttc ttt ttt gtc tct cag acc gct  276
Ala Leu Met Ser Pro Gly Pro Asp Phe Phe Phe Val Ser Gln Thr Ala
 15                  20                  25                  30
gtc agt cgt tcc cgt aaa gaa gcg atg atg ggc gtg ctg ggc att acc  324
Val Ser Arg Ser Arg Lys Glu Ala Met Met Gly Val Leu Gly Ile Thr
                 35                  40                  45
tgc ggc gta atg gtt tgg gct ggg att gcg ctg ctt ggc ctg cat ttg  372
Cys Gly Val Met Val Trp Ala Gly Ile Ala Leu Leu Gly Leu His Leu
             50                  55                  60
att atc gaa aaa atg gcc tgg ctg cat acg ctg att atg gtg ggc ggt  420
Ile Ile Glu Lys Met Ala Trp Leu His Thr Leu Ile Met Val Gly Gly
         65                  70                  75
ggc ctg tat ctc tgc tgg atg ggt tac cag atg cta cgt ggt gca ctg  468
Gly Leu Tyr Leu Cys Trp Met Gly Tyr Gln Met Leu Arg Gly Ala Leu
     80                  85                  90
aaa aaa gag gcg gtt tct gca cct gcg cca cag gtc gag ctg gcg aaa  516
Lys Lys Glu Ala Val Ser Ala Pro Ala Pro Gln Val Glu Leu Ala Lys
 95                  100                 105                 110
agt ggg cgc agt ttc ctg aaa ggt tta ctg acc aat ctc gct aat ccg  564
Ser Gly Arg Ser Phe Leu Lys Gly Leu Leu Thr Asn Leu Ala Asn Pro
                115                 120                 125
aaa gcg att atc tac ttt ggc tcg gtg ttc tca ttg ttt gtc ggt gat  612
Lys Ala Ile Ile Tyr Phe Gly Ser Val Phe Ser Leu Phe Val Gly Asp
            130                 135                 140
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aac gtt ggc act acc gcg cgc tgg ggc att ttt gcg ctg atc att gtc   660
Asn Val Gly Thr Thr Ala Arg Trp Gly Ile Phe Ala Leu Ile Ile Val
        145                 150                 155
gaa acg ctg gcg tgg ttt acc gtc gtt gcc agc ctg ttt gcc ctg ccg   708
Glu Thr Leu Ala Trp Phe Thr Val Val Ala Ser Leu Phe Ala Leu Pro
    160                 165                 170
caa atg cgc cgt ggt tat caa cgt ctg gcg aag tgg att gat ggt ttt   756
Gln Met Arg Arg Gly Tyr Gln Arg Leu Ala Lys Trp Ile Asp Gly Phe
175                 180                 185                 190
gcc ggg gcg tta ttt gcc gga ttt ggc att cat ttg att att tcg cgg   804
Ala Gly Ala Leu Phe Ala Gly Phe Gly Ile His Leu Ile Ile Ser Arg
                195                 200                 205
tgatgccaga cgcgtcttca gagtaagtcg gataag                          840
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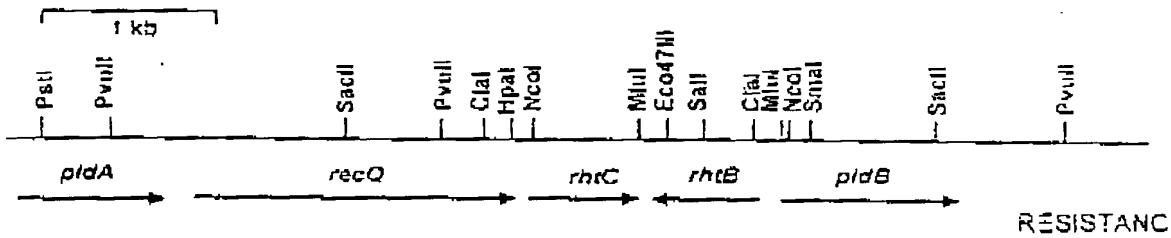
2. A method for producing an amino acid L-threonine or L-homoserine or L-valine or L-leucine , comprising the step of cultivating the bacterium belonging to the genus Escherichia, which has an ability to produce the amino acid, in a culture medium, to produce and accumulate the amino acid in the medium and recovering the amino acid from the medium, therein the bacterium Escherichia coli is used having an increased resistance to L-threonine which is determined by an increased content in cells of the RhtC protein encoded by the DNA fragment according to claim 1.

ABSTRACT

A DNA FRAGMENT rhtC ENCODING RhtC PROTEIN WHICH CONFERS ON BACTERIUM ESCHERICHIA COLI INCREASED RESISTANCE TO L-THREONINE, AND A METHOD FOR PRODUCING L-AMINO ACIDS

The present invention relates to biotechnology and genetic engineering. The application is made for a DNA fragment encoding rhtC protein which confers on bacterium Escherichia coli increased resistance to L-threonine. On the basis of the multicopy plasmid pRhtC, containing this fragment, the new E. coli strains were constructed: MG442/pRhtC-L-homoserine producer; and MG442/pVIC40, pRhtC-L-threonine prdocucer which are capable of increased producing of the above-mentioned amino acids as compared ot the strains not containing pRhtC plasmid. Besides, the E. coli strains NZ10/pRhtBC and and NZ10/pRhtB,pRhtC were obtained containing on the plasmids in addition to the rhtC gene also the rhtB gene which confers upon cells resistance to L-homoserine. These strains are capable of increased producing of L-homoserine, L-valine and L-leucine as compared to the strains not containing the above plasmids. Some physiological biochemical and cultural-morphological features of the new strains are described. A method for producing amino acid using the new strains is presented.



		RESISTANCE TO HOMOSERINE (10 mg/ml)	RESISTANCE TO THREONINE (50 mg/ml)
pRhtB	———	+	-
pNPZ44	———	-	+
pRhtBC	———	+	+
pRhtC	———	-	+

Fig. 1

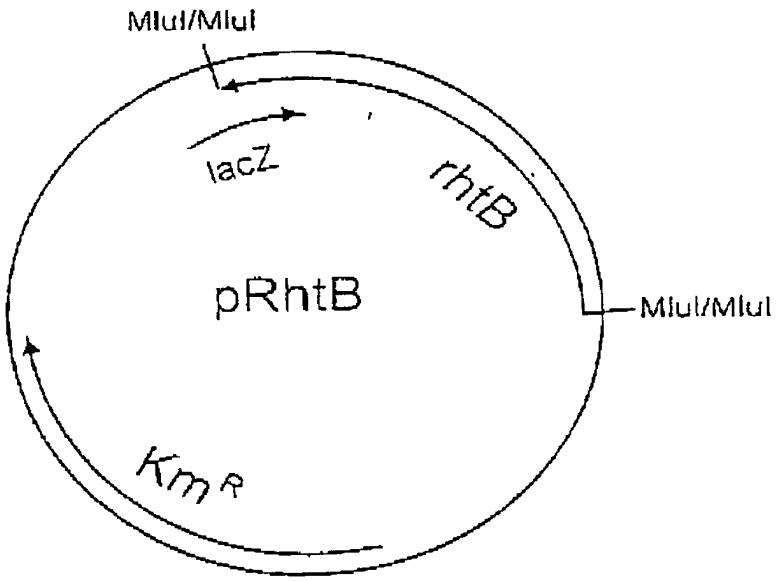


Fig. 2

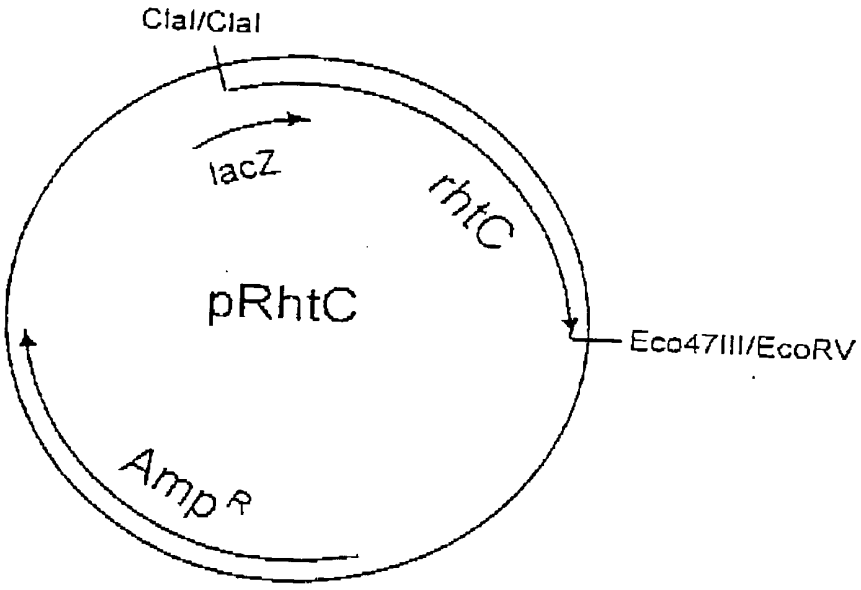


Fig. 3

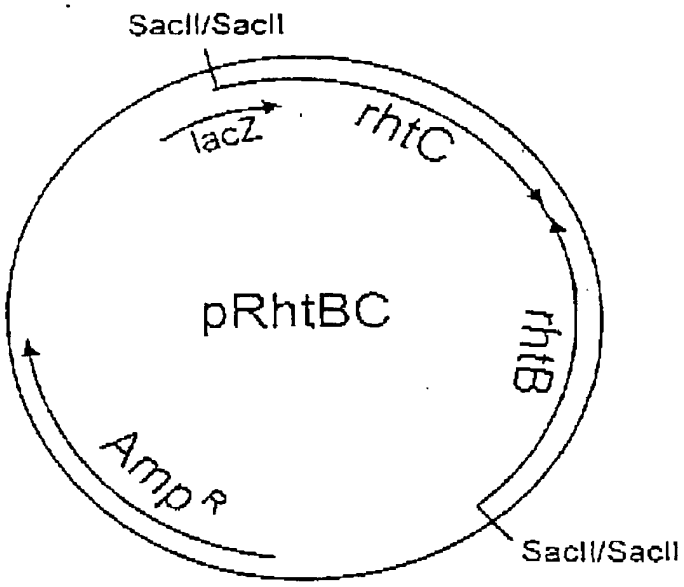


Fig. 4